BIOCONTROL OF TOBACCO MICROFLORA BY D-ALANINE

BIOCONTROLE DE LA MICROFLORE DU TABAC PAR LA D-ALANINE

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SUMMARY

The microbiological make-up of redried tobacco is almost exclusively made of bacteria from gram-positive Bacillus genus. On tobacco, most of the Bacillus cells are present in a dormant spore form.

A correlation was established between tobacco-dryer conditions and cigarette off-taste caused by microbial activation. Germination of dormant Bacillus spores and the subsequent release of dipicolinic acid seem to be involved in the mechanism resulting in the off-taste.

The transformation of Bacillus spores into vegetatively growing cells is a sequential process involving activation, germination and outgrowth. The key is the activation step which is a trigger reaction that conditions spores to germinate. It has been shown that the amino-acid L-alanine (L-ALA) naturally present in tobacco plays a predominant role in the germination process. L-ALA binds to a germination site at the surface of the spore, thus causing activation. A competitive inhibitor for the initiation of germination by L-ALA is D-alanine (D-ALA). The binding affinity of D-ALA to the germination site is stronger than that of L-ALA, so it is able to block the initiation of germination.

D-ALA was evaluated specifically as a control mechanism for the Bacillus spore population of tobacco. The efficacy of

D-ALA was quantified in tobacco extract against pure Bacillus pumilus spores - the predominant strain on tobacco - and evaluated on tobacco cut-filler by measuring germination yields under conditions favouring activation. Minimum germination inhibitory concentration was found around 200 ppm. Conditions favouring germination were simulated in semi-industrial trials with and without D-ALA added. The version treated with D-ALA was subjectively in line with a standard control, while subjective off-taste was detected on cigarettes prepared with untreated tobacco.

RESUME

La microflore du tabac reséché est composée presque exclusivement de bactéries à gram + appartenant au genre Bacillus. La majorité des cellules bactériennes présentes sur le tabac sont des spores dormantes.

Une corrélation a été établie entre les conditions de séchage et les cigarettes ayant un "off-taste" (faux goût) dû à une activation microbienne. La germination des spores de Bacillus et la libération d'acide dipicolinique semblent impliquées dans les mécanismes responsables d'un "off-taste".

On a démontré que la L-alanine (L-ALA), naturellement présente dans le tabac, joue un rôle prédominant dans le processus de germination. La L-ALA se fixe sur un site à la surface de la spore, activant ainsi la germination . La D-ALA, inhibiteur compétitif de la germination engendrée par la L-ALA, a une affinité pour le site de germination plus grande que celle de la forme L et peut ainsi bloquer la germination.

La D-ALA a été spécifiquement reconnue comme agent de contrôle de la population de spores de Bacillus présentes sur le tabac. Son efficacité a tout d'abord été quantifiée dans des extraits de tabac sur une culture pure de spores de B. pumilus, espèce prédominante du tabac. Son évaluation a ensuite été faite directement sur du tabac coupé, en

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mesurant le taux de germination dans des conditions favorisant une activation. La concentration minimale inhibant la germination fut estimée à environ 200 ppm. Des conditions favorisant la germination ont été simulées dans des essais semi-industriels en présence et en l'absence de D-ALA. Un "off-taste" subjectif fut détecté dans des cigarettes préparées avec du tabac non traité, alors que la version traitée avec de la D-ALA a été jugée en accord avec le standard utilisé comme contrôle.

TOBACCO MICROBIOLOGY

The microbial content of tobacco varies between one thousand and ten million organisms per gram, depending on tobacco type and origin. Three kinds of microorganisms can be isolated from tobacco : yeasts, molds and bacteria. Due to successive heat treatments in processing and low water activity, yeasts are rarely found and only a few molds can survive. The quasi-totality of the mold strains isolated and identified are characteristic members of the flora of the storage room : mainly Aspergillus and Penicillium. The only organisms that can easily survive the dessication process and heat treatments are the gram-positive spore formers Bacillus.

The microbial population of tobacco belongs exclusively (99.99%) to the Bacillus genus. Bacillus pumilus is the predominant strain found on tobacco. Bacillus subtilis and licheniformis are generally present at a few percent level as well as, sporadically, some other Bacillus stains.

A particularity of Bacillus is their ability to form spores considerably resistant to harsh environmental factors such as heat, dessication, toxic chemicals and a lack of nutrients over a long period of time.

The Bacillus life cycle involves sporulation, dormancy, spore germination, outgrowth and vegetative growth.

On tobacco, 80 to 100% of the Bacillus are present in the dormant form, the rest being germinated spores. During tobacco processing, a slight germination in the dormant spore population is naturally observed. This prefigures

that conditions favoring Bacillus spore germination, such as high water activity (AW) and heat-shock, may be encountered by tobacco. The transformation of Bacillus spores into vegetatively growing cells is a sequential process involving activation, germination and outgrowth. Activation is a trigger reaction conditioning spores to germinate.
On tobacco, the key factor for microbiological activity is water activity. Bacillus vegetative growth is only possible for AW above 0.95 (1). As AW never exceeds 0.8 at equilibrium during tobacco processing, the only risk of spoilage is limited to molds. Molds can develop on tobacco surface if its AW is maintained above 0.7 for several days. However, if no Bacillus growth is expected, conditions for spore germination - e.g., heat-shock and locally high AW at the tobacco surface - could be met for a short period in the process. Such conditions could be encountered in tobacco dryers where tobacco is heated in a saturated atmosphere.

CASE STUDY

The situation was illustrated by a cigarette off-taste problem detected after the cut-tobacco drying system was modified in a factory. Tobacco coming from cutters at a AW of 0.75 was divided into two dryers operating with different settings. The subjective evaluation of cigarettes made with tobacco from each dryer showed that one dryer was producing off-taste tobacco. It was established that the off-taste situation was caused by an excessive air temperature in the dryer.

Microbiological analyses revealed a significant difference between tobacco samples taken after each dryer. In the control situation, the total bacterial count was 9×10^5 with 85% of dormant spores, while in the off-taste conditions, the total population was reduced to 4.5×10^5 and the dormant spores to 30%. After that case, similar situations were analysed with the same microbiological profile, i.e., germination of dormant spores and reduction

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of the total population.

The mechanism of the off-taste was established. Specific conditions encountered in the dryer allow dormant spores to germinate. Entering outgrowth, cells losing osmo-tolerance during elongation are lysed, explaining population reduction. During germination, dipicolinic acid (DPA), the most abundant compound of the spore, is released (2). The direct implication of DPA (pyridine-2,6-dicarboxylic acid) in the off-taste was demonstrated by producing cigarettes with DPA levels in the range of what could be released by the natural tobacco spore population.
The subjective threshold limit established was between 100 and 500 ppb.

As germination activation is the critical step in the off-taste mechanism, the objective of the present study was to evaluate an integrated system inhibiting Bacillus spore germination.

PRINCIPLE

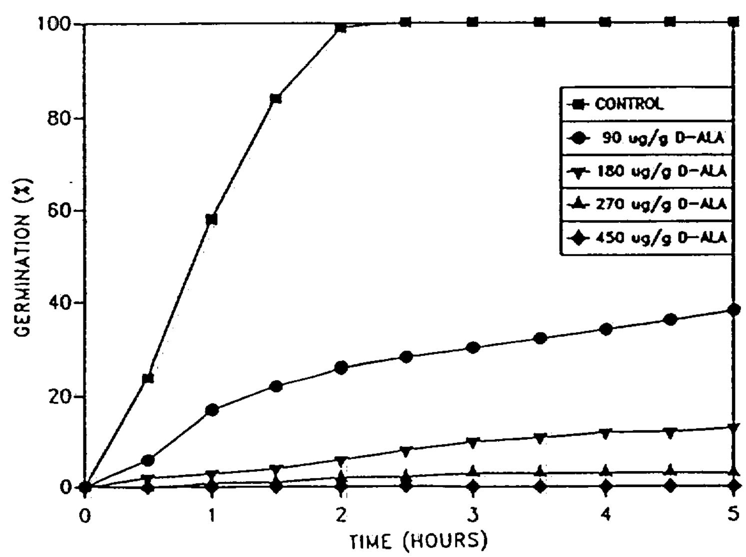
Beside AW and heat-shock, the amino-acid L-ALA - a physiological germinant naturally present on the tobacco - plays an important role in germination activation (3). A particularity of activation by L-ALA is a competitive inhibition of the reaction by the D-isomer : D-ALA.
The mechanism of action of both alanine enantiomers on germination is not yet clearly established (4). A proteinic receptor was localized at the surface of the spore. The allosteric site is hypothetically divided into two distinct portions. L-ALA may bind to the germination portion and activate germination mechanisms. The other portion is the inhibition part of the receptor. D-isomers of alanine and analogue amino-acids show a high affinity for this inhibition portion and can bind more strongly than L-ALA to the germination region. It was observed that the binding affinity of D-ALA is thirty times higher that that of L-ALA (5). Among all the analogues, D-ALA shows the highest affinity for the receptor and the strongest type of

inhibition of L-ALA germination activation (6). D-ALA was evaluated as an inhibitor of Bacillus spore germination in the tobacco context.

RESULTS AND DISCUSSION

In order to avoid problems caused by the heterogeneity of tobacco microflora, a pure culture of Bacillus pumilus spores was used as a first step, to quantify the effect of D-ALA on germination. Tobacco extracts were sterilized by successive filtrations, inoculated with B. pumilus spores and incubated at 37°C. Germination was followed by the loss of absorbance at 660 nanometers. Fig. 1 shows germination kinetics for increasing dosages of D-ALA (90 to 450 micro-grams per gram). In the control, germination is completed after 120 min. and then the cells start the growing process. Even with 90 ug D-ALA, the germination rate is seriously reduced. The minimal inhibitory concentration (MIC) was found to be around 200 ug/g.

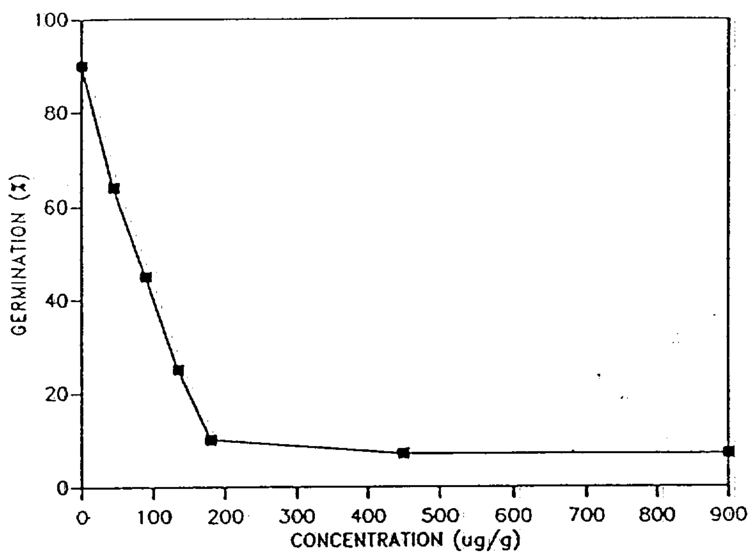
FIG. 1 INHIBITION OF BACILLUS PUMILUS SPORE GERMINATION IN TOBACCO EXTRACT



As a second step, D-ALA was tested directly on the tobacco, against its endogenous microflora, in conditions favoring Bacillus spore germination. D-ALA efficiency was evaluated

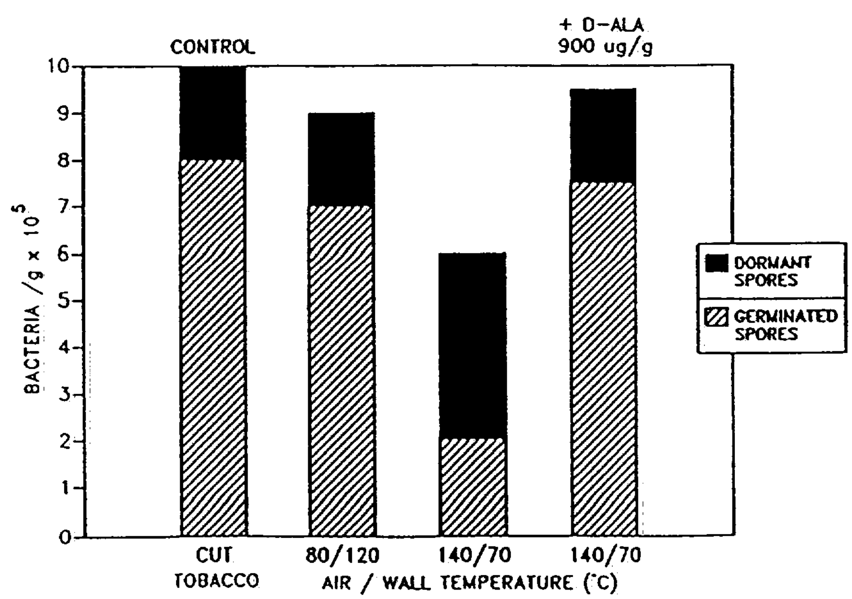
on cut-tobacco equilibrated at a AW of 0.98.
Fig. 2 presents the dose-response curve; as in tobacco extracts, the MIC is around 200 ug/g.

FIG. 2 INHIBITION OF SPORE GERMINATION BY D-ALA ON CUT TOBACCO EQUILIBRED AT AW 0.98



Finally, the effect of D-ALA in the off-taste situation was studied in pilot scale trials, by varying dryer-settings. The ratio of air temperature vs wall temperature was changed from 80°C/120°C to 140°C/70°C in the control, in order to induce spore germination and reproduce the off-taste problem (Fig. 3).

FIG. 3 SPORE GERMINATION IN CUT TOBACCO DRYER



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Tobacco treated with 900 ug/g D-ALA was evaluated in the same conditions. This time, the total number of bacteria as well as the spore proportion remained constant at the level of the cut-tobacco values. The subjective evaluation of the finished products showed a clear correlation between microbiological status and cigarette taste (Table 1).

TABLE 1
SUBJECTIVE EVALUATION OF CIGARETTES

VERSION	AIR / WALL TEMPERATURES	COMMENTS
CONTROL	80 / 120	Standard quality
TRIAL	140 / 70	Off-taste bitter, acidic
TRIAL + D-ALA	140 / 70	Standard quality

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